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of TAE684-induced apoptosis (Fig. 5C). These results thus suggested that inhibition of STAT3-survivin signaling by TAE684 contributes substantially to the induction of apoptosis by this drug. Collectively, our results thus suggested that inhibition of both the ERK-BIM and STAT3-survivin signaling pathways contributes to the induction of apoptosis associated with ALK inhibition in EML4-ALK–positive lung cancer cells.

Discussion

EML4-ALK was only recently identified as a transforming fusion gene in NSCLC (4). Although EML4-ALK was shown to possess marked oncogenic activity both *in vitro* and *in vivo* (4, 15), the signaling pathways underlying malignant transformation by the fusion protein have remained unclear. We have now shown that phosphorylation of both ERK and STAT3 was similarly and markedly increased in NIH 3T3 cells by forced expression of either variant 1 or variant 3 of EML4-ALK, whereas phosphorylation of AKT remained unaffected. Similar effects were observed in different clones of these cells stably transfected with a vector for either variant of EML4-ALK (data not shown). We further showed that the growth of both 3T3/EAV1 and 3T3/EAV3 cells was significantly attenuated by inhibition of ERK or STAT3 signaling but not by that of PI3K signaling. NPM-ALK has also been shown to activate ERK and STAT3 signaling pathways (6, 27–33), both of which are thought to be essential downstream mediators of the oncogenic action of NPM-ALK. In the present study, we found that ALK siRNA markedly abrogated the phosphorylation of AKT in the NPM-ALK–positive lymphoma cell line Karpas299, consistent with previous results implicating activation of PI3K-AKT signaling in malignant transformation by NPM-ALK (22–25). In contrast, we found that ALK siRNA did not suppress AKT phosphorylation in the EML4-ALK–positive lung cancer cell line H3122. Together, our results thus suggest that both ERK and STAT3 signaling pathways, rather than the PI3K signaling pathway, are the principal downstream pathways activated by EML4-ALK in lung cancer cells. Oncogenic ALK fusion proteins therefore may activate downstream pathways in a manner dependent on the fusion partner (Supplementary Fig. S1).

Preclinical studies have shown that treatment of NSCLC cell lines expressing EML4-ALK with ALK inhibitors suppresses cell proliferation and induces apoptosis (9, 34), although the underlying mechanisms of these effects were not well characterized. We have now shown that TAE684, a specific inhibitor of the kinase activity of ALK, significantly inhibited the phosphorylation of ERK and STAT3, but not that of AKT, in EML4-ALK–positive lung cancer cells, supporting the notion that ERK and STAT3 signaling pathways function downstream of EML4-ALK. BIM is a key proapoptotic member of the Bcl-2 family of proteins and initiates apoptosis signaling by binding to and antagonizing the function of prosurvival members of the Bcl-2 family (35). We found that TAE684 induced upregulation of BIM in EML4-ALK–positive lung cancer cells. With the use of RNAi-mediated depletion of ERK, we also found that BIM expression is regulated by the ERK signaling pathway. We further showed that knockdown of BIM by RNAi resulted in significant inhibition of TAE684-induced apoptosis in EML4-ALK–positive cells, suggesting that BIM induction mediated by inhibition of the ERK pathway plays a pivotal role in ALK inhibitor–induced apoptosis in EML4-ALK–positive lung cancer cells. These findings are consistent with the previous observation that inhibition of the ERK pathway contributes to EGFR-TKI–induced BIM upregulation, which is essential for the induction of apoptosis by these agents, in *EGFR* mutation–positive NSCLC cells (36–38).

Survivin is a member of the IAP family and protects against apoptosis by either directly or indirectly inhibiting the activation of effector caspases (39). We have now shown that TAE684 inhibited the expression of survivin in EML4-ALK–positive lung cancer cells. Furthermore, depletion of STAT3 resulted in downregulation of survivin expression, whereas expression of a constitutively active form of STAT3 resulted in upregulation of survivin expression. These data indicate that expression of survivin is regulated primarily through the STAT3 signaling pathway, consistent with the results of a previous study (40). We further found that expression of CA-STAT3 blocked the TAE684-induced downregulation of survivin, indicating that ALK inhibition results in survivin downregulation through inhibition of the STAT3 signaling pathway. Forced expression of either CA-STAT3 or survivin attenuated TAE684-induced apoptosis in 3T3/EAV3 or H3122 cells, suggesting that inhibition of STAT3-survivin signaling contributes to ALK inhibitor–induced apoptosis in EML4-ALK–positive lung cancer cells. Our present data thus suggest that ALK inhibitor–induced apoptosis is mediated both by upregulation of BIM through inhibition of the ERK pathway and by downregulation of survivin through inhibition of the STAT3 pathway in EML4-ALK–positive lung cancer cells.

In conclusion, our results have identified both ERK and STAT3 signaling pathways as key mediators of the transforming activity of EML4-ALK in lung cancer cells positive for this fusion protein. We further demonstrated that inhibition of both ERK-BIM and STAT3-survivin signaling pathways is responsible for ALK inhibitor–induced apoptosis in these cells. Our results thus provide a basis for the further development of ALK-targeted therapy in EML4-ALK–positive lung cancer patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 2004;350:2129–39.
2. Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 2004;304:1497–500.
3. Pao W, Miller V, Zakowski M, Doherty J, Politi K, Sarkaria I, et al. EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. Proc Natl Acad Sci U S A 2004;101:13306–11.
4. Soda M, Choi YL, Enomoto M, Takada S, Yamashita Y, Ishikawa S, et al. Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. Nature 2007;448:561–6.
5. Morris SW, Kirstein MN, Valentine MB, Dittmer KG, Shapiro DN, Saltman DL, et al. Fusion of a kinase gene, ALK, to a nucleolar protein gene, NPM, in non-Hodgkin's lymphoma. Science 1994;263:1281–4.
6. Fujimoto J, Shiota M, Iwahara T, Seki N, Satoh H, Mori S, et al. Characterization of the transforming activity of p80, a hyperphosphorylated protein in a Ki-1 lymphoma cell line with chromosomal translocation t(2;5). Proc Natl Acad Sci U S A 1996;93:4181–6.
7. Inamura K, Takeuchi K, Togashi Y, Nomura K, Ninomiya H, Okui M, et al. EML4-ALK fusion is linked to histological characteristics in a subset of lung cancers. J Thorac Oncol 2008;3:13–7.
8. Inamura K, Takeuchi K, Togashi Y, Hatano S, Ninomiya H, Motoi N, et al. EML4-ALK lung cancers are characterized by rare other mutations, a TTF-1 cell lineage, an acinar histology, and young onset. Mod Pathol 2009;22:508–15.
9. Koivunen JP, Mermel C, Zejnullahu K, Murphy C, Lifshits E, Holmes AJ, et al. EML4-ALK fusion gene and efficacy of an ALK kinase inhibitor in lung cancer. Clin Cancer Res 2008;14:4275–83.
10. Shinmura K, Kageyama S, Tao H, Bunai T, Suzuki M, Kamo T, et al. EML4-ALK fusion transcripts, but no NPM-, TPM3-, CLTC-, ATIC-, or TFG-ALK fusion transcripts, in non-small cell lung carcinomas. Lung Cancer 2008;61:163–9.
11. Martelli MP, Sozzi G, Hernandez L, Pettirossi V, Navarro A, Conte D, et al. EML4-ALK rearrangement in non-small cell lung cancer and non-tumor lung tissues. Am J Pathol 2009;174:661–70.
12. Shaw AT, Yeap BY, Mino-Kenudson M, Digumarthy SR, Costa DB, Heist RS, et al. Clinical features and outcome of patients with non-small-cell lung cancer who harbor EML4-ALK. J Clin Oncol 2009;27:4247–53.
13. Wong DW, Leung EL, So KK, Tam IY, Sihoe AD, Cheng LC, et al. The EML4-ALK fusion gene is involved in various histologic types of lung cancers from nonsmokers with wild-type EGFR and KRAS. Cancer 2009;115:1723–33.
14. Sasaki T, Rodig SJ, Chirieac LR, Janne PA. The biology and treatment of EML4-ALK non-small cell lung cancer. Eur J Cancer 2010;46:1773–80.
15. Choi YL, Takeuchi K, Soda M, Inamura K, Togashi Y, Hatano S, et al. Identification of novel isoforms of the EML4-ALK transforming gene in non-small cell lung cancer. Cancer Res 2008;68:4971–6.
16. Soda M, Takada S, Takeuchi K, Choi YL, Enomoto M, Ueno T, et al. A mouse model for EML4-ALK-positive lung cancer. Proc Natl Acad Sci U S A 2008;105:19893–7.
17. Kwak EL, Bang YJ, Camidge DR, Shaw AT, Solomon B, Maki RG, et al. Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. N Engl J Med 2011;363:1693–703.
18. Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Pestell RG, Albanese C, et al. Stat3 as an oncogene. Cell 1999;98:295–303.
19. Okamoto K, Okamoto I, Okamoto W, Tanaka K, Takezawa K, Kuwata K, et al. Role of survivin in EGFR tyrosine kinase inhibitor-induced apoptosis in EGFR mutation-positive non-small cell lung cancer. Cancer Res 2010;70:10402–10.
20. Okamoto W, Okamoto I, Tanaka K, Hatashita E, Yamada Y, Kuwata K, et al. TAK-701, a humanized monoclonal antibody to HGF, reverses gefitinib resistance induced by tumor-derived HGF in non-small cell lung cancer with an EGFR mutation. Mol Cancer Ther 2010;10:2785–92.
21. Tanaka K, Arao T, Maegawa M, Matsumoto K, Kaneda H, Kudo K, et al. SRPX2 is overexpressed in gastric cancer and promotes cellular migration and adhesion. Int J Cancer 2009;124:1072–80.
22. Bai RY, Ouyang T, Miething C, Morris SW, Peschel C, Duyster J. Nucleophosmin-anaplastic lymphoma kinase associated with anaplastic large-cell lymphoma activates the phosphatidylinositol 3-kinase/Akt antiapoptotic signaling pathway. Blood 2000;96:4319–27.
23. Polgar D, Leisser C, Maier S, Strasser S, Ruger B, Dettke M, et al. Truncated ALK derived from chromosomal translocation t(2;5)(p23;q35) binds to the SH3 domain of p85-PI3K. Mutat Res 2005;570:9–15.
24. Slupianek A, Nieborowska-Skorska M, Hoser G, Morrione A, Majewski M, Xue L, et al. Role of phosphatidylinositol 3-kinase-Akt pathway in nucleophosmin/anaplastic lymphoma kinase-mediated lymphomagenesis. Cancer Res 2001;61:2194–9.
25. Rassidakis GZ, Feretzaki M, Atwell C, Grammatikakis I, Lin Q, Lai R, et al. Inhibition of Akt increases p27Kip1 levels and induces cell cycle arrest in anaplastic large cell lymphoma. Blood 2005;105:827–9.
26. Galkin AV, Melnick JS, Kim S, Hood TL, Li N, Li L, et al. Identification of NVP-TAE684, a potent, selective, and efficacious inhibitor of NPM-ALK. Proc Natl Acad Sci U S A 2007;104:270–5.
27. Crockett DK, Lin Z, Elenitoba-Johnson KS, Lim MS. Identification of NPM-ALK interacting proteins by tandem mass spectrometry. Oncogene 2004;23:2617–29.
28. Marzec M, Kasprzycka M, Liu X, Raghunath PN, Wlodarski P, Wasik MA. Oncogenic tyrosine kinase NPM/ALK induces activation of the MEK/ERK signaling pathway independently of c-Raf. Oncogene 2007;26:813–21.
29. Amin HM, McDonnell TJ, Ma Y, Lin Q, Fujio Y, Kunisada K, et al. Selective inhibition of STAT3 induces apoptosis and G(1) cell cycle arrest in ALK-positive anaplastic large cell lymphoma. Oncogene 2004;23:5426–34.
30. Zamo A, Chiarle R, Piva R, Howes J, Fan Y, Chilosi M, et al. Anaplastic lymphoma kinase (ALK) activates Stat3 and protects hematopoietic cells from cell death. Oncogene 2002;21:1038–47.
31. Shi X, Franko B, Frantz C, Amin HM, Lai R. JSI-124 (cucurbitacin I) inhibits Janus kinase-3/signal transducer and activator of transcription-3 signalling, downregulates nucleophosmin-anaplastic lymphoma kinase (ALK), and induces apoptosis in ALK-positive anaplastic large cell lymphoma cells. Br J Haematol 2006;135:26–32.
32. Han Y, Amin HM, Franko B, Frantz C, Shi X, Lai R. Loss of SHP1 enhances JAK3/STAT3 signaling and decreases proteosome degradation of JAK3 and NPM-ALK in ALK +anaplastic large-cell lymphoma. Blood 2006;108:2796–803.
33. Chiarle R, Simmons WJ, Cai H, Dhall G, Zamo A, Raz R, et al. Stat3 is required for ALK-mediated lymphomagenesis and provides a possible therapeutic target. Nat Med 2005;11:623–9.
34. McDermott U, Iafrate AJ, Gray NS, Shioda T, Classon M, Maheswaran S, et al. Genomic alterations of anaplastic lymphoma kinase may sensitize tumors to anaplastic lymphoma kinase inhibitors. Cancer Res 2008;68:3389–95.
35. Chen L, Willis SN, Wei A, Smith BJ, Fletcher JI, Hinds MG, et al. Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. Mol Cell 2005;17:393–403.
36. Costa DB, Halmos B, Kumar A, Schumer ST, Huberman MS, Boggon TJ, et al. BIM mediates EGFR tyrosine kinase inhibitor-induced apoptosis in lung cancers with oncogenic EGFR mutations. PLoS Med 2007;4:1669–79; discussion 1680.
37. Cragg MS, Kuroda J, Puthalakath H, Huang DC, Strasser A. Gefitinib-induced killing of NSCLC cell lines expressing mutant EGFR requires BIM and can be enhanced by BH3 mimetics. PLoS Med 2007;4:1681–89; discussion 1690.
38. Gong Y, Somwar R, Politi K, Balak M, Chmielecki J, Jiang X, et al. Induction of BIM is essential for apoptosis triggered by EGFR kinase inhibitors in mutant EGFR-dependent lung adenocarcinomas. PLoS Med 2007;4:e294.
39. Hengartner MO. The biochemistry of apoptosis. Nature 2000;407:770–6.
40. Aoki Y, Feldman GM, Tosato G. Inhibition of STAT3 signaling induces apoptosis and decreases survivin expression in primary effusion lymphoma. Blood 2003;101:1535–42.